Journal of Chromatography, 261 (1983) 407–414
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,717

OFF-LINE LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC STUDIES OF *o*-PHTHALALDEHYDE–PRIMARY AMINE DERIVATIVES

RICHARD C. SIMPSON*, JIMMY E. SPRIGGLE and HANS VEENING*
Department of Chemistry, Bucknell University, Lewisburg, PA 17837 (U.S.A.)
(Received January 18th, 1983)

SUMMARY

The structures of the products formed between several *n*-aliphatic amines and *o*-phthalaldehyde (OPT) during routine post-column derivatization after high-performance liquid chromatographic (HPLC) elution, were determined by gas chromatography–mass spectrometry. OPT derivatization studies were carried out separately in the presence of mercaptoethanol (MCE) and in the presence of ethanethiol (ETH). Comparison of the mass spectra of the silylated OPT–MCE derivatized amines with those of the non-silylated OPT–ETH derivatives indicated a consistent fundamental structure with similar fragmentation patterns. It was shown that the structure of the fluorescent product obtained under analytical post-column HPLC derivatization conditions is a 1-alkylthio-2-alkyl-substituted isoindole. This finding is in agreement with results reported earlier for preparative-scale reactions.

INTRODUCTION

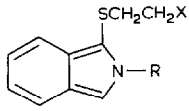
High-performance liquid chromatographic (HPLC) separations of simple aliphatic amines pose a special problem involving detection. The amines do not absorb in the UV region of the spectrum nor do they possess native fluorescence. Therefore, it is necessary to derivatize these compounds to a more easily detected form. Derivatization usually involves chromophoric or fluorophoric labeling of the molecule prior to detection. The advantage of using the latter method lies in the superior specificity and sensitivity of fluorescence detection over ultraviolet (UV) or visible (VIS) absorption[1].

Various reagents are currently available for fluorescent labeling of primary amines. These include fluorescamine[2,3], dansyl chloride[4,5], and the increasingly popular reagent, *o*-phthalaldehyde (OPT). Since OPT was first introduced by Roth[6], it has been used in a wide variety of applications[7–9]. In 1976, Simons and Johnson[10] presented the first study elucidating the structure of the fluorescent product formed in the

* Present address: Department of Chemistry, University of Rhode Island, Kingston, RI 02881, U.S.A.

0021-9673/83/$03.00

preparative reaction of a primary amine with OPT and 2-mercaptoethanol (MCE). Based upon data collected from 100-MHz proton NMR spectra of derivatized *n*-butylamine, they concluded that the structure of the product was a 1-alkylthio-2-alkyl-substituted isoindole[10,11], as shown in Fig. 1. In a later publication, they demonstrated that ethanethiol (ETH) could replace MCE to yield a more stable product of the same general structure[12].



X = OH for 2-mercaptoethanol
X = H for ethanethiol

Fig. 1. Structure of the derivative as proposed by Simons and Johnson[10,11].

In a later investigation by Simons and Johnson[13] they reported results based on the UV spectra of the derivatization reaction mixture of various primary amines to confirm the presence of the isoindole ring system. They also performed mass spectral (MS) analysis of various amines derivatized on a preparative scale in order to establish further the structure of the fluorescent product. However, due to the difficulty in isolating isoindoles[13], several measures, including long reaction times (15 min), the use of bulky alkyl thiols and low temperatures of crystallization (0°C), were taken to permit precipitation and isolation of the fluorescent adduct. Although these conditions did achieve preparative isolation of the product, they are not typical of those normally used in OPT-derivatization of primary amines in HPLC applications. Normally, on-line HPLC procedures are carried out over a much shorter period of time (less than 1 min) in alkaline aqueous buffers, which are at or above room temperature. Thus, it is not necessarily valid to assume that the fluorescent products, obtained through the use of each of these vastly different conditions, are the same.

The purpose of our study was to determine the structure of the major product obtained for amines actually eluted from HPLC columns and subjected to post-column derivatization. This was achieved by MS analysis of extracts of the derivatization reaction mixtures. Various aliphatic primary amines were derivatized with OPT and MCE and again with OPT and ETH. Thus, the structure of the product was observed as a function of amine structure and as a function of using either ETH or MCE. The results obtained were then cross-checked against each other to establish a consistent structure of the fluorescent adduct.

We have presented detailed MS fragmentation patterns for several of the primary amine–OPT derivatives in this paper. The availability of such data for these important HPLC derivatives should be useful as the trend toward combined HPLC–MS continues to grow.

EXPERIMENTAL

Apparatus

The HPLC system was a Spectra-Physics SP-8700 solvent delivery system (Spectra-Physics, Santa Clara, CA, U.S.A.) equipped with a Model 7120 injection

valve (Rheodyne, Cotati, CA, U.S.A.), fitted with a 100-μl sample loop. The column used was 30 cm × 3.9 mm I.D., packed with Zorbax C_8 bonded phase material (DuPont, Wilmington, DE, U.S.A.). The post-column OPT derivatizing reagent was pumped into the column effluent by a Model 39-650 high pressure pump (Rainin Instrument, Woburn, MA, U.S.A.) through a Swagelok "T" connector (Allentown Valve and Fitting, Emmaus, PA, U.S.A.). On-line post-column derivatization occurred in a reaction coil which was 305 cm × 0.023 mm I.D. coiled stainless steel tubing (Supelco, Bellefonte, PA, U.S.A.). The fluorophores were detected by a Schoeffel FS-970 spectrofluoromonitor (Kratos, Westwood, NJ, U.S.A.) with the excitation monochromator set at 340 nm and equipped with a Type 440 emission filter (Kratos).

The gas chromatograph–mass spectrometer was a Finnigan Model 4021 (Finnigan, Sunnyvale, CA, U.S.A.). The gas chromatograph was equipped with a 25 m × 0.25 mm I.D. SE-54 (film thickness 0.25 μm) fused silica column (J & W Scientific, Rancho Cordova, CA, U.S.A.).

Reagents

Aliphatic amines (methylamine, 40% aqueous; ethylamine, 70% aqueous; *n*-propylamine, 98%; *n*-butylamine, 96%; *n*-amylamine, 99%), ETH and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Aldrich (Milwaukee, WI, U.S.A.). OPT was purchased from Sigma (St. Louis, MO, U.S.A.). Reagent grade boric acid and anhydrous sodium sulfate were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Matheson, Coleman & Bell (Norwood, OH, U.S.A.) was the source of reagent grade MCE. HPLC grade methylene chloride and methanol were purchased from Burdick & Jackson Lab. (Muskegon, MI, U.S.A.). Ultrapure water was produced in-house by a Milli-Q reagent grade water system (Millipore, Bedford, MA, U.S.A.). All other incidental chemicals were of reagent grade purity.

The first derivatizing reagent, OPT–MCE, was prepared by dissolving 800 mg of OPT and 600 μl of MCE in 10 ml of methanol; this mixture was then added to approximately 950 ml of a 0.50 *M* potassium borate buffer at pH 9.0. The total volume was then brought to 1.00 l by addition of more borate buffer. The second derivatizing reagent, OPT–ETH, was prepared identically. Multicomponent amine mixtures were prepared by mixing 1-ml samples of each amine and shaking thoroughly.

Procedure

A 100-μl sample of the five component amine mixture was injected into the HPLC system and eluted through the column with 0.20 *M* sodium acetate buffer (pH 4.50) at a flow-rate of 2.0 ml/min. The OPT–MCE (or OPT–ETH) reagent was added to the column effluent at a rate of 0.7 ml/min. The detector effluent was collected for the duration of full-scale detector response. The collected fraction was then extracted with two 2-ml portions of methylene chloride. The methylene chloride layer was removed and rendered anhydrous by being passed through a short Pasteur pipette containing a plug of glass wool and anhydrous sodium sulfate. The resulting methylene chloride fraction was collected and evaporated under a stream of dry nitrogen, to a volume of approximately 100 μl. The OPT–MCE derivatized amines were then further derivatized for GC–MS analysis by adding 100 μl of BSTFA to the methylene

chloride concentrate, mixing thoroughly and warming gently for 1 min to insure complete silylation. This part of the procedure was omitted for the OPT–ETH derivatized amines. A 0.1-μl aliquot of either the silylated methylene chloride concentrate, or the untreated OPT–ETH sample was injected into the GC–MS system at an injector temperature of 310°C, using a 15:1 split ratio and a helium carrier flow-rate of 1 ml/min. The column temperature was initially held at 100°C for 2 min, then increased to 300°C at a rate of 15°C/min for OPT–MCE derivatives and 12°C/min for OPT–ETH derivatives. The mass spectrometer was used in the electron-impact (EI) mode, with the electron energy set at 70 eV. The scan time was 1 sec over a mass range of m/z 33 to m/z 500.

RESULTS AND DISCUSSION

The purpose of the current HPLC–MS study was to expose aliphatic amines to conditions actually encountered in a typical HPLC separation and on-line post-column derivatization, even though minimal separation of these compounds was achieved by reversed-phase HPLC. Each of the amines was derivatized with OPT–MCE and OPT–ETH in separate experiments. This permitted observation of the derivative structures for each amine as a function of either MCE or ETH addition to the OPT derivatizing reagent. Due to the presence of a hydroxyl group in the derivative formed with OPT–MCE (see Fig. 1), it was necessary to silylate the anhydrous extract concentrate of the on-line HPLC derivatization reaction in order to obtain a product which was sufficiently volatile to survive gas chromatographic (GC) separation.

The reconstructed ion current gas chromatogram (RIC) of the silylated extract of the OPT–MCE derivatization is shown in Fig. 2. The peaks at scan numbers 722,

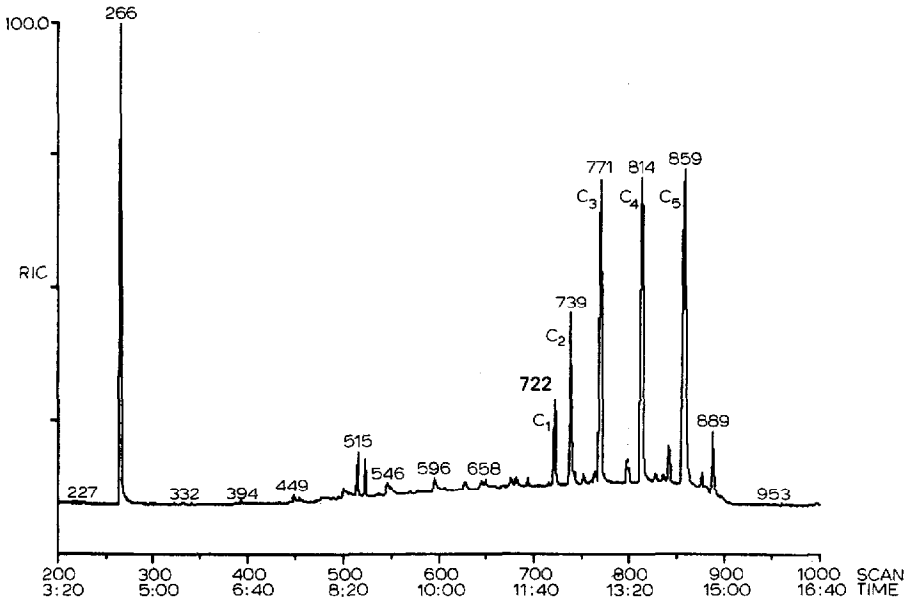


Fig. 2. RIC of silylated OPT–MCE amine derivatives.

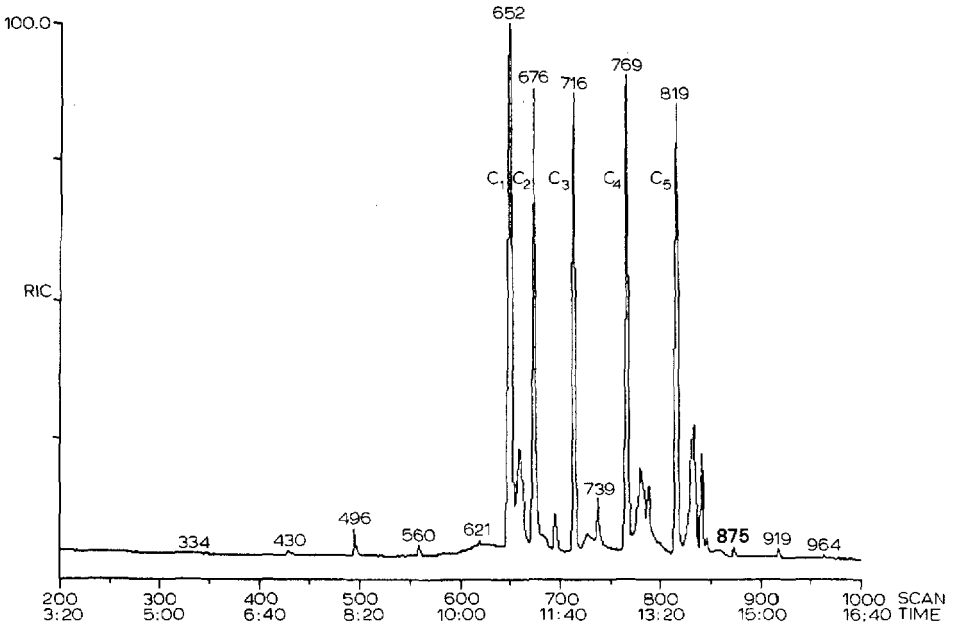


Fig. 3. RIC of OPT–ETH amine derivatives.

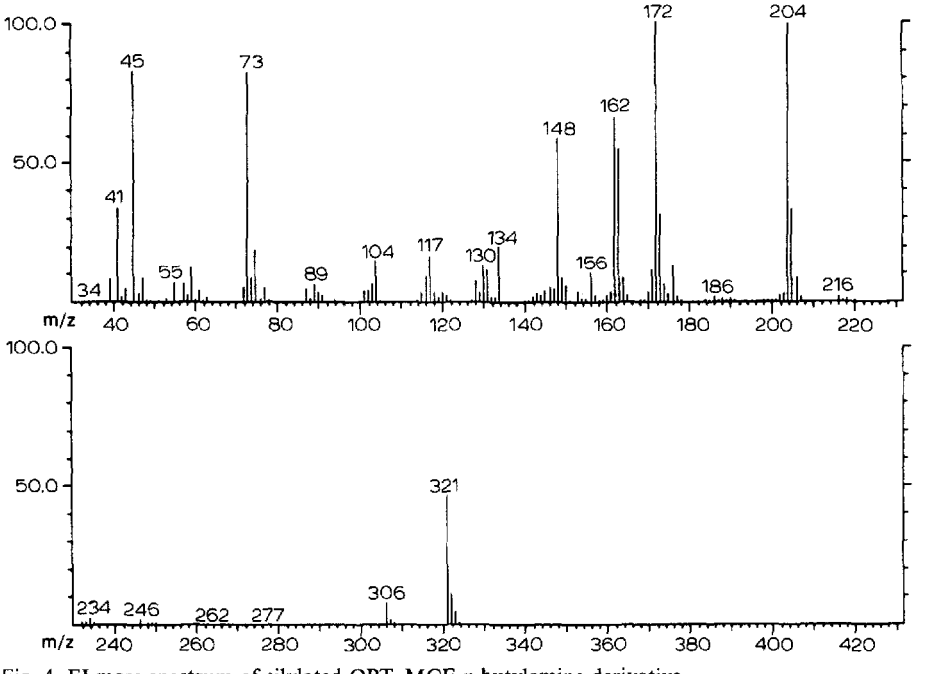
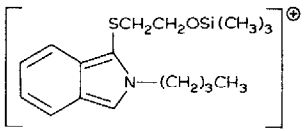
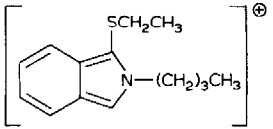
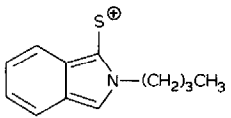
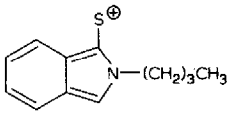
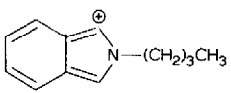
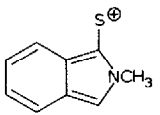
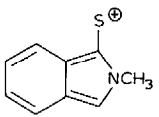
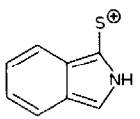
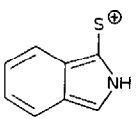


Fig. 4. EI mass spectrum of silylated OPT–MCE *n*-butylamine derivative.

TABLE I

EI MASS SPECTRAL FRAGMENTATION OF *n*-BUTYLAMINE DERIVATIVES

OPT–MCE n-butylamine derivative (silylated)			*OPT–ETH n-butylamine derivative (unsilylated)*		
m/z	*Rel. int. (%)*	*Assigned fragment*	*m/z*	*Rel. int. (%)*	*Assigned fragment*
321 (M^+)	46		233 (M^+)	68	
204	99		204 (base)	100	
172 (base)	100		162	82	
162	66		148	86	
148	59				

739, 771, 814 and 859 represent the silylated forms of derivatized methyl-, ethyl-, *n*-propyl-, *n*-butyl- and *n*-amylamine, respectively. Fig. 3 is the RIC of the products resulting from on-line HPLC derivatization with OPT–ETH. The peaks at 652, 676, 716, 769 and 819 represent the derivatives obtained for methyl-, ethyl-, *n*-propyl-, *n*-butyl- and *n*-amylamine, respectively. Other peaks in both chromatograms (Figs. 2 and 3) are due to derivatized isomeric amines, incompletely derivatized amines, impurities and decomposition products.

In this discussion, only the structural analysis of derivatized *n*-butylamine will be presented; although, the structures for the other four derivatized amines were found to be analogous. The EI mass spectra of the silylated OPT–MCE and the OPT–ETH derivatives of *n*-butylamine are shown in Figs. 4 and 5, respectively. In Fig. 4, the molecular ion (M^+) and the base peak were observed at *m/z* 321 and *m/z* 172, respectively. Fig. 5 showed M^+ at *m/z* 233 and the base peak at *m/z* 204. Table I lists the major *m/z* values, their relative MS peak intensities and the assigned molecular fragments. The other analogous silylated OPT–MCE and unsilylated OPT–ETH amine derivatives exhibited similar patterns consistent with their respective molecular weights. Table II summarizes the *m/z* values of the respective M^+ ions and base peaks from the mass spectrum for each amine derivatized by both methods.

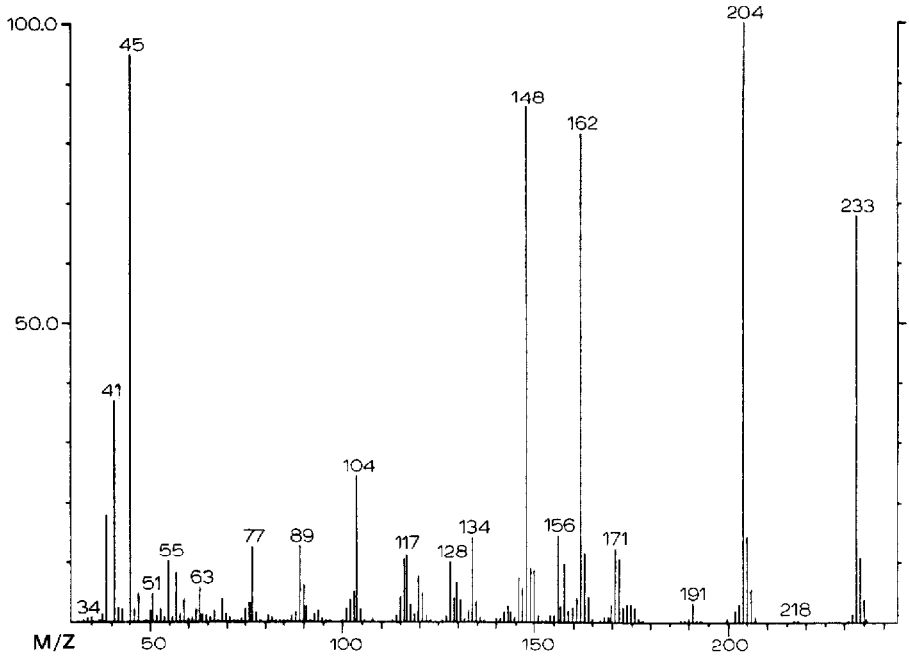


Fig. 5. EI mass spectrum of OPT–ETH *n*-butylamine derivative.

Examination of the data in Table I suggests a consistent basic structure of the derivative, regardless of which reagent was used to produce it. The difference in the M^+ *m/z* values is due to the presence of a trimethylsilylated hydroxyl group in the OPT–MCE derivative which is absent in the OPT–ETH derivative, thus resulting in a consistent difference of 88 *m/z* units for the two respective molecular ions for each

TABLE II

SUMMARY OF MS DATA FOR C_1–C_5 DERIVATIZED ALIPHATIC AMINES

Parent amine	*OPT–MCE derivative (silylated)*		*OPT–ETH derivative (unsilylated)*	
	M^+ *(m/z)*	*Base peak (m/z)*	M^+ *(m/z)*	*Base peak (m/z)*
Methylamine	279	162	191	162
Ethylamine	293	176	205	146
n-Propylamine	307	190	219	190
n-Butylamine	321	172	233	204
n-Amylamine	335	186	247	162

amine. The data presented in Table II, along with more complete fragmentation patterns not presented in this paper, show that the fundamental structure of OPT–MCE or OPT–ETH amine derivatives was independent of the amine used.

This study has demonstrated that under conditions normally used for HPLC on-line post-column derivatization of amines: (a) no preparative isolation of the product is necessary for structural analysis; (b) the fundamental structure of the fluorophore was consistently present in all derivatives; (c) substitution of ETH for MCE in the OPT reagent gives an analogous non-hydroxylated derivative (as expected) and (d) the structure of the fluorescent product obtained under analytical post-column derivatization HPLC conditions is a 1-alkylthio-2-alkyl-substituted isoindole, in agreement with that reported in the literature for preparative-scale reactions.

ACKNOWLEDGEMENTS

We thank the donors of the Petroleum Research Fund, administered by the American Chemical Society; the National Institute of Arthritis, Metabolism and Digestive Diseases (Grant AM25785); and the Camille and Henry Dreyfus Foundation for financial support.

REFERENCES

1 E. Johnson, A. Abu-Shumays and S. R. Abbott, *J. Chromatogr.*, 134 (1977) 107.
2 M. Kai, T. Ogata, K. Haraguchi and Y. Ohkura, *J. Chromatogr.*, 163 (1979) 151.
3 A. Licht, R. L. Bowman and S. Stein, *J. Liq. Chromatogr.*, 4 (1981) 825.
4 M. M. Abdel-Monem and K. Ohno, *J. Chromatogr.*, 107 (1975) 416.
5 N. D. Brown, R. B. Sweet, J. A. Kintzios, H. D. Cox and B. P. Doctor, *J. Chromatogr.*, 164 (1979) 35.
6 M. Roth, *Anal. Chem.*, 43 (1971) 880.
7 F. Perini, J. B. Sadow and C. V. Hixson, *Anal. Biochem.*, 94 (1979) 431.
8 J. C. Hodgin, *J. Liq. Chromatogr.*, 2 (1979) 1047.
9 R. C. Simpson, H. Y. Mohammed and H. Veening, *J. Liq. Chromatogr.*, 5 (1982) 245.
10 S. S. Simons, Jr. and D. F. Johnson, *J. Am. Chem. Soc.*, 98 (1976) 7098.
11 S. S. Simons, Jr. and D. F. Johnson, *J. Chem. Soc. Chem. Commun.*, (1977) 374.
12 S. S. Simons, Jr. and D. F. Johnson, *Anal. Biochem.*, 82 (1977) 250.
13 S. S. Simons, Jr. and D. F. Johnson, *J. Org. Chem.*, 43 (1978) 2886.